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Production of 1,3-propanediol from glycerol under haloalkaline conditions by halanaerobium hydrogeniformans

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PRODUCTION OF 1,3-PROPANEDIOL FROM GLYCEROL UNDER HALOALKALINE CONDITIONS BY *HALANAEROBIUM HYDROGENIFORMANS*

by

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This thesis has been prepared in the style of two journals. The first, a review within the literature review section has been prepared for submission to *Current Biotechnology*. A second manuscript has been prepared for submission to *Extremophiles*. Pages 4-16 are prepared for submission as a review. Pages 28-35 are prepared for submission as a journal article to *Extremophiles*. Pages 1-3, 17-27, and 36-39 are included as the standard thesis preparation.

ABSTRACT

With increased demands around the world to make modern lifestyles more environmentally friendly, the chemical commodity market has rapidly shifted. Through new technologies in chemical production, certain high value products have oversaturated the market and have become high-volume, low value waste products. The expansion of biodiesel production offers a prime example; high volumes of glycerol byproduct from this process have shifted glycerol from a high priced commodity to a common waste product. A number of microorganisms are known to synthesize the polymeric precursor 1,3-propanediol from glycerol; however, crude glycerol from biodiesel production creates a harsh environment for most microbes, and must go through expensive pre-treatment steps to lower alkaline pH values and salt concentrations before it can be considered a suitable feedstock. Halanaerobium hydrogeniformans has been identified to convert glycerol into 1,3-propanediol under haloalkaline conditions. Samples were grown over five days at pH 11 and at 7% (w/v) NaCl. The growth medium was amended with vitamin B12 to stimulate 1,3-propanediol production. HPLC analysis indicated statistically significant production of 1,3-propanediol, with the vitamin B12 amended bottles having a significant increase in 1,3-propanediol production compared to glycerol-only cultures. Data indicated a 0.6mol/mol conversion for vitamin B12 amended samples, while glycerol-only cultures had a conversion rate of 0.32mol/mol. H. hydrogeniformans, and potentially other haloalkaliphilic bacteria, provides a unique opportunity to develop new chemical processes that can overcome traditional problems and increase profitability by reducing the need for pH neutralization and dilution of residual salts in wastes such as crude glycerol.

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

1. INTRODUCTION

1.1. BACKGROUND

For thousands of years, microbiology has been vital to industry. The first examples were the brewing of beer and wine leading to immense tax revenues in the ancient word. Now, everyday life is intertwined with industrial microbiology. From the food you eat, to the fuel in your car, microbiology is everywhere.

With the development of new molecular techniques and sequencing technologies, industrial microbiology and biotechnology has grown immensely. Week after week, during the previous twenty years, reports were released detailing new processes with exotic microbes, newly optimized processes due to better understanding of metabolisms, and record breaking yields due to developments in engineering processes. With these technological advancements, a new avenue of approach has recently been examined in using bacteria suited for extreme environments to bypass steps within industrial processes to reduce costs, save energy, and push the envelope of what is possible in biotechnology.

During the 1960s, Thomas D. Brock and Hudson Freeze were conducting studies in the hot springs at Yellowstone National Park. Up until this time, the only work on extreme organisms had been focused on spore forming *Bacillus* and fungus. In 1969, Brock and Freeze published an article on the isolation of a hyperthermophile, *Thermus aquaticus* [1]. This study was very different from earlier work with thermophiles, as *T. aquaticus* required temperatures above 70°C to grow and thrive, as opposed to the traditional thermophilic enrichment at 55°C that had been unsuccessful for the enrichment of organisms other than *Bacillus*. Twenty years later, *T. aquaticus* was recognized for potentially being useful, when its DNA polymerase was isolated by Kary Mullis to develop the polymerase chain reaction technique, and changing molecular biology forever.

From that first major study by Brock and Freeze, studies on extremophilic microbiology have included every extreme condition one could imagine. From acidic conditions and heavy metal contamination, to alkalinity and salinity, through

temperatures spanning sub-freezing to boiling, microbes have been found to live in almost any environment imaginable. With these unique adaptations, extremophiles have become of great interest to industry.

Two of the most significant and expensive conditions for microbial-based processes are the presence of high salt concentrations and significant pH extremes in process feedstocks. Haloalkaliphiles are extremophilic bacteria that can survive in high salt concentrations and at high pH. Many of these organisms are found in diverse microbial ecosystems and have a wide range of unique properties and metabolisms. The organism of interest for this project is *Halanaerobium hydrogeniformans*. Originally isolated from Soap Lake, Washington, *H. hydrogeniformans* has been observed to have a few special characteristics. Most notably, *H. hydrogeniformans* can produce large amounts of hydrogen gas, comparable to genetically engineered organisms [2]. This process is important for the production of biohydrogen as a fuel. However, the focus for this thesis work was to identify ways to convert industrial waste into useful products by using this organism.

One of the biggest sources of industrial waste in contemporary industrial biotechnology is the excess glycerol produced during biodiesel synthesis. The effluent from this process contains crude glycerol and has salt concentrations in excess of 3% and pH at or above 10 [3]. Identifying new markets and developing processes for the utilization of crude glycerol waste products is imperative for the efficient and economical production of biodiesel. Preliminary analysis of the genome of *Halanaerobium hydrogeniformans* indicated that the bacterium contained genes associated with glycerol metabolism, with the ability to potentially form ethanol, hydrogen, 1,3-propanediol, and 1-propanol as fermentation products [4]. After the initial genome review, glycerol fermentation would be tested under haloalkalline conditions. First, *H. hydrogeniformans* would need to be grown in the presence of glycerol, to identify growth capability and rate. Next, the fermentation products would need to be identified to determine if any value added products were being produced during growth. Finally, an attempt was made to stimulate and increase production of the desired fermentation products. The preliminary analysis indicated that 1,3-propanediol, a valuable commodity used heavily

in industrial polymer synthesis, should be produced as glycerol is fermented by *H. hydrogeniformans*.

1.2. OBJECTIVES AND GOALS

1.2.1. Identify Glycerol Metabolism of *H. hydrogeniformans.* The first objective was to identify the glycerol metabolism capability of *Halanaerobium hydrogeniformans* and identify the possible end products of glycerol fermentation through HPLC analysis. The growth rate of the organism was examined along with HPLC analysis used to define glycerol uptake and product formation rates.

1.2.2. Identify Tolerances of *H. hydrogeniformans.* The second objective was to examine the tolerances of *Halanaerobium hydrogeniformans* to salt and alkalinity values that match what is found in waste glycerol. In addition, both the tolerance to glycerol concentrations along with the tolerance of the organism to 1,3-propanediol were examined.

1.2.3. Measure and Optimize 1,3-Propanediol Production. The third objective was to characterize the 1,3-propanediol production pathway, optimize production, and examine the minimums and maximums of production. The effects of vitamin B_{12} concentration on 1,3-propandiol production was examined. HPLC analysis was used to measure the production of 1,3-propanediol.

PAPER

1. METABOLIC CAPABILITIES OF THE MEMBERS OF THE ORDER HALANAEROBIALES AND THEIR POTENTIAL BIOTECHNOLOGICAL APPLICATIONS

To be submitted for the special issue "Biotechnological Applications of Extremophiles" for Current Biotechnology

1.1. ABSTRACT

The order Halanaerobiales contains a number of well-studied halophiles that possess great potential biotechnological application. The unique halophilic adaptations that these organisms utilize, such as intercellular increase of KCl, combined with their ability to ferment simple sugars, provides an excellent platform for biotechnological development over a wide range of salt levels and other extreme conditions. From fermented foods to oil reservoirs, members of Halanaerobiales have been found in many environments. The environmental conditions many of these organisms grow provide excellent analogs to industrially important processes, such as alkaline pre-treated biomass stocks, treatment of crude glycerol from biodiesel production, salty fermented foods, and bioremediation of contaminants under extreme conditions of salinity and in some cases, alkalinity. From heat and salt stable enzymes to waste fermentations, bioremediation options, bioenergy, and MEOR, Halanaerobiales provides a wide spectrum of environmentally friendly solutions to current problems.

Keywords:

Bioenergy, bioremediation, fermented foods, Halanaerobiales, halophilic hydrolases, MEOR

1.2. INTRODUCTION

The order of Halanaerobiales is composed of anaerobic Gram negative, rod-shaped bacteria that are halotolerant to halophilic. Though not as well characterized as members of the Clostridiales order, and specifically the Clostridia, members of the Halanaerobiales possess many unique physiological adaptations and applications that can be utilized for biotechnological purposes. For example, most of the organisms in this order have a salting-in mechanism to combat the osmotic stresses of halophilic environments. Since potassium is accumulated in the cell, these organisms must possess salt tolerant proteins, thus, providing a promising opportunity to identify and isolate halotolerant enzymes. Furthermore, the central metabolic processes of these organisms tend to be simple sugar fermentations or homoacetogenesis. Understanding these metabolic pathways can potentially lead to more efficient biomass pretreatment and fermentation with applications towards biofuel production.

1.3. CHARACTERIZATION OF THE MEMBERS OF THE HALANAEROBIALES

The order Halanaerobiales is in the Clostridia class of the Firmicutes phylum. There are two families within the Halanaerobiales order, the Halobacteroidaceae and the Halanaerobiaceae. There are currently four recognized genera in the Halanaerobiaceae family. The genus, *Halanaerobium*, has nine validly published species (*H. acetethylicum*, *H. alcaliphilum*, *H. congolense*, *H. fermentans*, *H. kushneri*, *H. lacurosei*, *H. praevalens*, *H. saccharolyticum*, and *H. salsuginis*), two subspecies of *H. saccarolyticm*, (subspecies *saccharolyticum* and *senegalense*) and one species that has not been validly published yet, *H. hydrogeniformans*. Four other genera have been placed into this order, (*Halarsenatibacter*, *Halocella*, *Haloincola*, and *Halothermothrix*). The family Halobacteroidaceae contains 11 genera, (*Acetohalobium*, *Fuchsiella*, *Halanaerobacter*, *Halanaerobaculum*, *Halanaerocella*, *Halobacteroides*, *Halonatronum*, *Natroniella*, *Orenia*, *Selenihalanaerobacter*, and *Sporohalobacter*). Most all of the

species, characterized to date, ferment sugars. However, there are a few exceptions. For example, *Acetohalobium arabaticum* and *Fuchsiella alkaliacetigena* are acetogens and are able to form acetate from H_2 and CO_2 [1],[2]. *Natroniella sulfidigena* exhibits no fermentative growth but is able to respire with sulfur/polysulfide or fumarate [3]. This organism is also able to grow autotrophically with either H2 or formate [3]. A number of other members of the order Halanerobiales are also capable of respiration. *Selenihalanaerobacter shriftii* can respire with selenate or nitrate [4] and *Halarsenatibacter silvermanii* can respire with arsenate, Fe (III) or elemental sulfur [5]. *Halanaerobium conglense* is capable of sugar fermentation or respiration with either thiosulfate or elemental sulfur [6]. Thus, this group of organisms possesses varied metabolisms that can potentially be used for purposes ranging from food preparation [7], [8] to bio-energy production [9], [10].

1.4. FOOD FERMENTATION AND PREPARATION

The first description of a novel halophilic anaerobe from a food product was the isolation and characterization of the organism *Halanaerobium fermentans* from fermented puffer fish ovaries. Key data from this experiment was the large production of organic acids by this organism. Many of these acids are essential to the fermentation process, and help in imparting the characteristic fermentation flavor to many of these dishes [7]. The same research group also found another *Halanaerobium* species, *H. praevalens*, present in Swedish fermented herrings. The isolates in these experiments produced large amounts of organic acids, specifically acetic and butyric acid [8]. Another fermented fish dish, funazushi (fermented Crucian carp with rice), was found to possess *Halanaerobium* species after a 90-day fermentation [11]. As concluded by the authors of the funazushi study, these organisms may be key in developing the flavor and texture of fermented seafoods. Other salty fermented foods might also benefit from the ability of these organisms to ferment a variety of carbohydrates. For example, Cho and Seo [12] found *Halanaerobium* species 16S RNA genes present in soybean-fermented foods such as doenjang (soybean paste) and ganjang (soybean sauce) while they were investigating the microbial diversity in traditional food substances by using a PCR-based approach.

1.5. CHEMICAL FERMENTATIONS

Glycerol is a common osmoregulatory compound produced by *Dunaliella*, a unicellular green algae that is found in saline environments [13]. It would be suspected that many organisms isolated from saline environments would be capable of utilizing this compound. Surprisingly, only three organisms, *Halanaerobacter jeridensis* [14]. *Halanaerobium hydrogeniformans* [15] and *Halanaerobium saccharolyticum* subsp. *saccharolyticum* [16] in this order have been reported to ferment glycerol. The potential for using bacteria to ferment glycerol to valuable products is becoming more attractive. This metabolic process was reported in *Clostridium acetobutylicum* and other strains of *Clostridium* and noted that it could be of importance to the chemical industry [17]. With the increase in biodiesel production around the world, waste glycerol has become a topic of concern for many bioenergy companies. One of the most profitable ways to treat waste glycerol is to use a biological process to convert the glycerol into 1,3-propanediol. 1,3-propanediol is commonly used as a polymer for paints, adhesives, and fragrances. Kivisto et al. [16] found that *Halanaerobium saccharolyticum* subsp. *saccharolyticum* can produce 1,3-propanediol from glycerol under halophilic conditions. They obtained an approximately a 60% mol to mol conversion of glycerol to 1,3-propanediol with supplementation of 64 µg/L of Vitamin B_{12} at pH of 7.8. Additionally, *Halanaerobium hydrogeniformans* has been shown to produce 1,3-propanediol under haloalkaline conditions. Roush et al., [15] were able to demonstrate production of 1,3-propanediol at pH 11 and 7% NaCl (w/v). It is important to note that crude glycerol generally has a high pH as well as containing salt [15]. In contrast to Kivisto et al. [16], *H. hydrogeniformans* did not appear to have a variance of production while varying the B_{12} concentration, with a conversion of about 55% over 48 hours (unpublished, manuscript under preparation). Further research will enable the optimization of this process and possible commercialization.

1.6. ISOLATION OF ENZYMES SUCH AS HYDROLASES

There is great interest in finding hydrolytic enzymes, such as carbohydrate hydrolases, that are reactive under extreme environments. A key characteristic of the Halanaerobiales is that most of the members of this order use a "salting-in" mechanism to regulate the osmotic pressure of their saline environments [18]. The organisms accumulate potassium ions to counter balance the sodium ions present. Thus, the enzymes present in these organisms will tend to be more salt-tolerant than enzymes retrieved from other organisms.

Halothermothrix orenii is one of the few identified thermophilic members of Halanaerobiales [19]. It has been the target of most of the research conducted on Halanaerobiales has been focused on this organism, because temperature and salt stable enzymes are of extreme important to industry. The AmyB gene from *H. orenii* was characterized to have an optimum activity at 0.9M NaCl, with 12% activity at 4.3M NaCl and 45% activity in the absence of NaCl. In regards to temperature optimums, this enzyme is most active at 65°C [20].

Halocella cellulolytica was identified through a phylogenetic study to have cellulase activity. *Halocella cellulolytica* was able to digest cellulose, at an optimum NaCl concentration of 15%, and produce ethanol and hydrogen [21]. Further studies have been conducted to enrich for the cellulases from this organism, due to the biotechnological implications of having a salt stable cellulase [22].

1.7. BIOENERGY

Many of these organisms produce fermentation products that can be used as fuel sources such as ethanol and hydrogen under saline and possibly alkaline conditions. Due to this versatility, these organisms can possibly be used over a wide range of conditions and lead to the development of biotechnologies in response to the ever-changing range of biomass feedstock.

Biological hydrogen production is one of the targets for bioenergy companies, auto manufacturers and researchers. Hydrogen is an energy dense fuel that is relatively easy to transport and inexpensive to produce. Two organisms within this order have been discovered to produce industrially significant amounts of hydrogen gas, *Halanaerobium saccharolyticum* [23] and *Halanaerobium hydrogeniformans* [9]. Kivistö et al., [16, 23] have studied hydrogen production from *Halanaerobium saccharolyticum* from the fermentation of glycerol. Two subspecies of *Halanaerobium saccharolyticum* were examined, *saccharolyticum* and *senegalensis*. *Saccharolyticum* was able to produce a 0.6mol/mol ratio at 15% (w/v) NaCl and pH 7.4, while *senegalensis* was able to produce 1.6mol/mol hydrogen at 15% (w/v) NaCl and pH 7.0.

Hydrogen production with *Halanaerobium hydrogenformans* was demonstrated by growing the organism on alkaline pre-treated straw and switchgrass as well as glucose and cellobiose [9]. Optimal hydrogen production from 15 mMcellobiose occurred at a NaCl concentration of 7% and a pH of 11 with a hydrogen molar yield of 2.3.

Work has also been performed utilizing an unidentified *Halanaerobium* species in a microbial fuel cell. Paul [24] demonstrated that the organisms attached to the anode and obtain current densities up to 12.5mA/m^2 and open circuit voltage up to 1V. Though the power production was very low, this research does demonstrate the possibility of using similar organisms, especially those that possess cytochromes, for biofuel cell applications under saline and alkaline conditions.

1.8. BIOREMEDIATION

The use of a co-culture of *Halanaerobium spp.* with CO_2-utilizing halophiles may provide an opportunity to sequester CO_2 as acetate in salt caverns [24]. Salt caverns can be an expensive storage area for use in CO_2 sequestration, however, they are the only option in some areas for handling this greenhouse gas. Additionally, these caverns have high salinity and temperatures, further limiting the types of organisms that can be used. However, an unidentified *Halanaerobium* species was found to make up at least 15.3% of the genomic DNA found in some of these sites. It is anticipated that with proper

enrichment, these organisms could lead to CO_2 sequestration methods utilizing bacterial communities found within the caverns [24].

Research has also indicated that members of Halanaerobiales, specifically *Halanaerobium* species, are capable of removing organic carbon from saline wastewater. Researchers were able to remove 94% of the chemical oxygen demand (COD) at an initial COD of 1900mg/L, with a 19h hydraulic retention time and 3% salt. As the salt concentration increased beyond 3%, COD removal became less efficient. It is interesting to note that at 3% salt concentration, the optimum COD depletion occurred, a similar salinity to seawater [26, 27].

In regards to the biodegradation of hazardous contaminants, *Halanaerobium praevalens* and *Sporohalobacter marismortui* have been shown to degrade nitro-substituted aromatic compounds, such as nitrobenzene, nitrophenols, and nitroaniline within 24 hours, at concentrations up to 100mg/L. The researchers found that optimal rates of degradation occurred at concentrations of approximately 50mg/L and that larger concentrations inhibited growth [28]. More work needs to be performed to determine if other members of this group are capable of degrading hazardous contaminants.

1.9. MEOR

Microbial enhanced oil recovery (MEOR) has been a topic of research for years. Most contemporary research involves stimulating latent organisms to produce surfactants and other biomolecules to stimulate the release of oil [29]. It is possible to utilize gas production to dissolve carbonates and assist with the movement of trapped crude. Gasses suggested for this process are methane, carbon dioxide, and hydrogen gas. Organisms in the order of Halanaerobiales are excellent candidates for MEOR, as they anaerobically ferment simple sugars, and produce essential MEOR products. They also have been identified in oil wells, can be enriched for easily and do not consume the hydrocarbons, or produce hydrogen sulfide [6, 30, 31, 32, 33]. For example, *Halanaerocella petrolearia* discovered in oil reservoirs possesses optimal growth conditions of 15 NaCl (w/v), temperature between 40-45°C and, as a simple sugar fermenter, can produce

abundant amounts of carbon dioxide and hydrogen gas. Carbon dioxide is important as it can raise the pressure within the wells, and help increase crude yield. Hydrogen gas combined with an aqueous environment can dissolve the carbonate rocks, changing the pore size and increasing yield [33].

1.10. CONCLUSIONS

From *H. pravaelens* providing umami and sour flavors in fermented foods, continuing to chemical synthesis and bioenergy production by *H. hydrogeniformans*, *Halanaerobiales* contains many organisms with a variety of applications. Two species of *Halanaerobium* and one species of *Halanaerobacter* ferment glycerol. Using halotolerant organisms to in 1,3-propanediol production allows for higher concentrations of crude glycerol to be used in the process along with being able to avoid having to neutralize and purify the feedstock, thereby saving both energy and money over methods based around *Klebsiella* and *E. coli*.

The breakdown of carbohydrate feedstocks in an industrial setting requires steps of neutralization, desalination and temperature control. *H. orenii* provides a unique opportunity to harvest hydrolases that can withstand both increased NaCl concentration and increased thermal requirements. *H cellulolytica* has been identified to have halotolerant cellulase activity. Bioenergy production, H. hydrogeniformans and *H. saccharolyticum* both can produce significant amounts of hydrogen gas, similar to genetically engineered *Clostridia* species. However, due to haloalkaliphilic and halotolerant capabilities, these organisms can produce fuel in pretreated but non-neutralized feedstocks, saving energy and capital.

Halophiles, and *Halanaerobiales* specifically, are becoming an ever growing area of interest for industrial microbiology and biotechnology. Further characterization will take place due to the ever growing suite of genomic tools and myriad sequencing projects, leading to an expanded understanding of *Halanaerobiales* and an even greater expansion of the biotechnological niches of the members of this order.

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SECTION

2. REVIEW OF LITERATURE

2.1. GLYCEROL TO 1,3-PROPANEDIOL CONVERSION

2.1.1. The Pathway. Production of 1,3-propanediol in microorganisms is linked to glycerol metabolism. When glycerol is taken up by the cell, it has multiple fates. Glycerol can be converted into dihydroxyacetone and then integrated into pyruvate metabolism, or glycerol is converted into 1,3-propanediol to replenish NAD^+. During typical glycerol metabolism, excess NADH is produced by the cell. Much of the NADH produced is recycled to NAD^+ through the formation of fermentation end products. However, some NAD^+ must be replenished through an alternate pathway. Excess glycerol is shunted into the 1,3-propanediol production pathway where NADH is utilized to form 1,3-propanediol. Figure 2.1 shows the pathway from *Clostridium butyricum* [22].

The first step in the conversion of glycerol to 1,3-propanediol is the removal of a water molecule from glycerol by the enzyme glycerol dehydratase (E.C. 4.2.1.30). This step creates the intermediate 3-hydroxypropanal. Next, the enzyme 1,3-propanediol dehydrogenase (E.C. 1.1.1.202), uses an NADH to form 1,3-propanediol, replenishing the NAD^+ needed by the cell for normal metabolism [5].

The first step in the pathway, catalyzed by glycerol dehydratase, requires a Vitamin B_{12} cofactor. Supplementation of the growth media with Vitamin B_{12} can cause a shift in the production and growth of the organism to dramatically increase 1,3-propanediol yields while affecting the organism growth rate [5].

Another property of this pathway is the growth-linked nature of 1,3-propanediol production. Streekstra et al. (1987) were able to show this fermentation property in *Klebsiella aerogenes.* The work demonstrated that glycerol fermentation to 1,3-propanediol was growth linked and 1,3-propanediol could inhibit growth in high concentrations. They were also able to show that fast growing cells produced greater

amounts of 1,3-propanediol than slow growing cells, matching up with the NADH linked pathway [6].

In 1990, Homann et al. showed that both *Citrobacter* and *Klebsiella* strains could ferment glycerol to 1,3-propanediol. Their work focused on enriching for 1,3-propanediol producing strains, and to try to identify the differences in fermentation pathways. *Klebsiella* utilizes external electron acceptors, like nitrate and nitrite, to regenerate NAD^+, while *Citrobacter* uses the same fermentation pathway as *Clostridia* and *Halanaerobium* species [7].

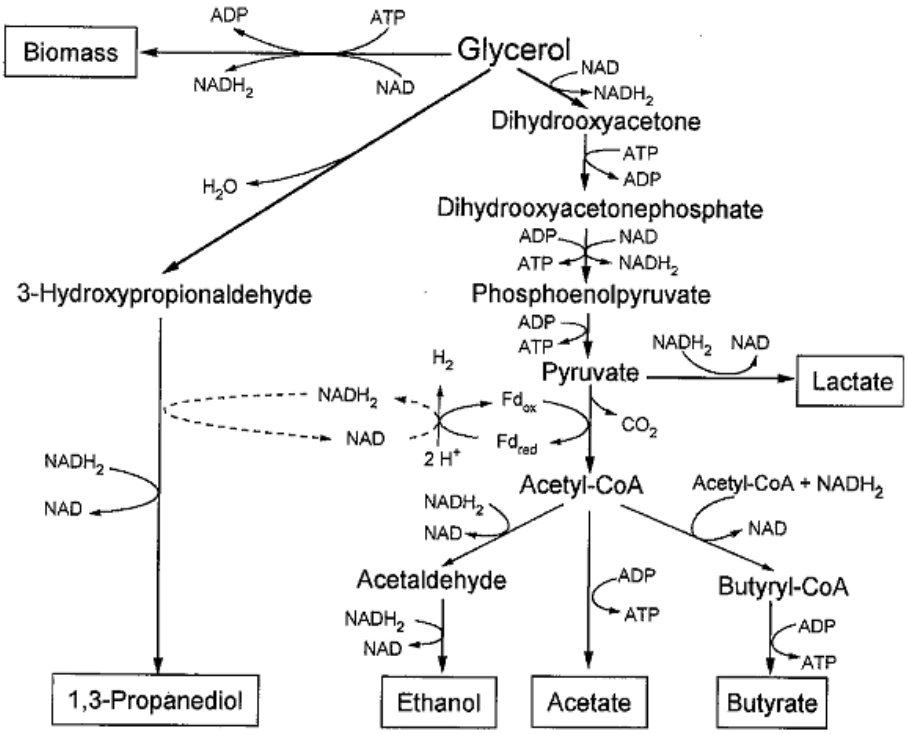

Figure 2.1. 1,3-Propanediol Pathway adapted from [22].

2.1.2. Biodiesel and Crude Glycerol. Biodiesel is one of the most successful first generation biofuels. The most common way to produce biodiesel is the base-catalyzed tranesterification method. Alcohol and catalyst are mixed together, and then mixed with oils and fats. The most desirable oils are that of vegetable varieties, that allow for a renewable source of oils for the reaction [8]. Figure 2.2 is a flow chart detailing this process.

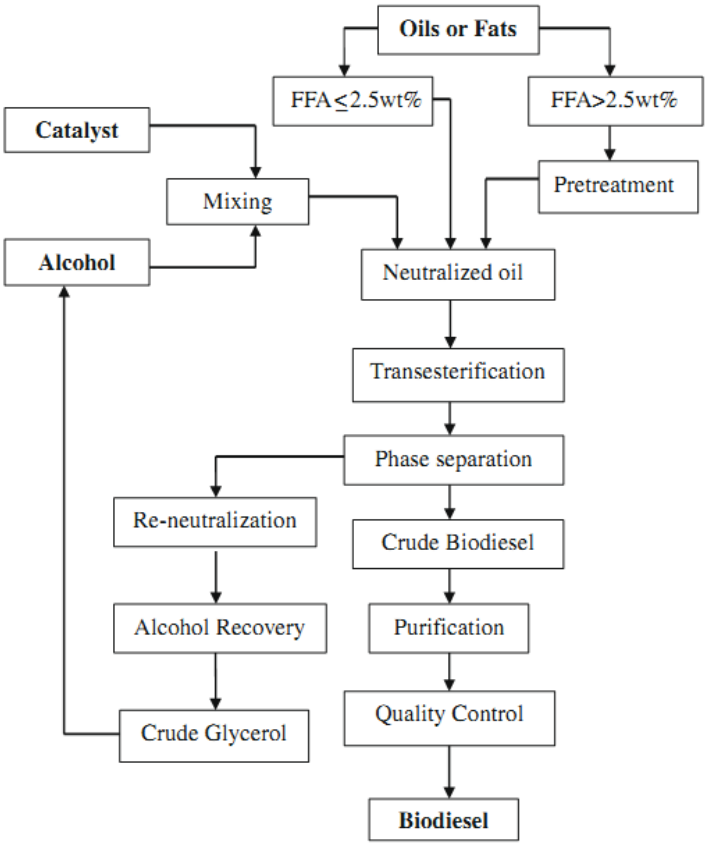

Figure 2.2. Flowchart detailing biodiesel synthesis as modified from [25].

After the reaction is complete, the resulting products are biodiesel and crude glycerol. Crude glycerol contains many of the byproducts of the reaction like soap, along with catalysts and contaminants. Sodium methoxide is the catalyst most used in this process and with the alcohol component being methanol, one could imagine the harshness of crude glycerol on a living cell. The concentrations of these aforementioned chemicals can vary widely. Glycerol concentrations range from 38% to 96%, with methanol concentrations as high as 14% and sodium content approaching 5% [3]. These concentrations make it difficult to develop a biological process that does not involve neutralizing the crude glycerol or diluting it heavily. However the use of haloalkaliphiles provide a unique opportunity to bypass traditional neutralization steps, and feed the crude glycerol directly to the organisms.

2.1.3. Current Technologies for 1,3-Propanediol Production. The rapid increase in biodiesel production in the past decade has led to a projected surplus of over six times demand by 2020 [9]. This has led to many researchers to determine how to produce a commodity out of this waste product. Much have this research has been focused on a handful of bacteria genera. These include *Clostridia*, *Klebsiella*, and *Halanaerobium*, along with engineered *Clostridia butyricum* and *Escherichia coli*.

Clostridia were some of the organisms initially studied when examining this conversion, along with *Klebsiella* and *Citrobacter*. Biebl et al. (1992) were able to enrich from environmental samples a *Clostridia* species that could convert glycerol to 1,3-propanediol at a .51mol/mol ratio [10]. Four years later, Zeng conducted kinetic analysis of the *Clostridia butyricum* conversion pathway and concluded that the maximum conversion of 64% when the organism is producing carbon dioxide and hydrogen gas during fermentation, and 71% when the sole fermentation products are 1,3-propanediol and butyric acid [5].

Another organism that has been the focus of continued research is *Klebsiella*. Early work in 1987 by Streekstra focused on the kinetics of the conversion process in bioreactors [6]. Three years later, Homann et al. showed that both *Klebsiella* and *Citrobacter* could produce appreciable amounts of 1,3-propanediol from glycerol [7]. In the 2000s, new combination fermentation techniques were incorporated into processes

with *Klebsiella* [11]. These combined anaerobic/aerobic processes have lower total conversions compared to dedicated anaerobic fermentations, however they do not require the supplementation of vitamin B_{12} and carry out the conversion reactions at a higher rate [12].

Up until the past three years, much of the focus has been on well-known fermenting organisms. However, extremophilic fermenters have been discovered that can carry out this process. The Kivistö group has been working with *Halanaerobium saccharolyticum,* a halotolerant bacteria that can produce large amounts of hydrogen and 1,3-propanediol, similar to *H. hydrogeniformans*. The group was able to demonstrate that *H. saccharolyticum* could convert glycerol to 1,3-propanediol at a conversion ratio of .60 mol/mol at 7% NaCl concentrations. However, this required the supplementation of vitamin B_{12} of 64μg/L leading to a process that is not economical [13].

Finally, there has been some work conducted attempting to genetically engineer strains of *C. butyricum* and *E. coli* to overproduce 1,3-propanediol. These efforts have been met with great success. In 2009, Tang et al., were able to engineer a strain of *E. coli* to convert glycerol to 1,3-propanediol at a ratio of 90% (g/g). They used a two-step fermentation method previously done with *Klebsiella* and a dramatic temperature shift from 30°C to 42°C [14]. Later, Otte et al. used a technique called genomic shuffling to improve strains of *Clostridia diolis* for 1,3-propanediol production. They were able to improve yields from the wild type strain by 80%, bringing the yield to a 61% conversion. The group also was able to grown the engineered strains on crude glycerol and obtain yields of 49% conversion [15]. Figure 2.3 illustrates a comparison between these current technologies.

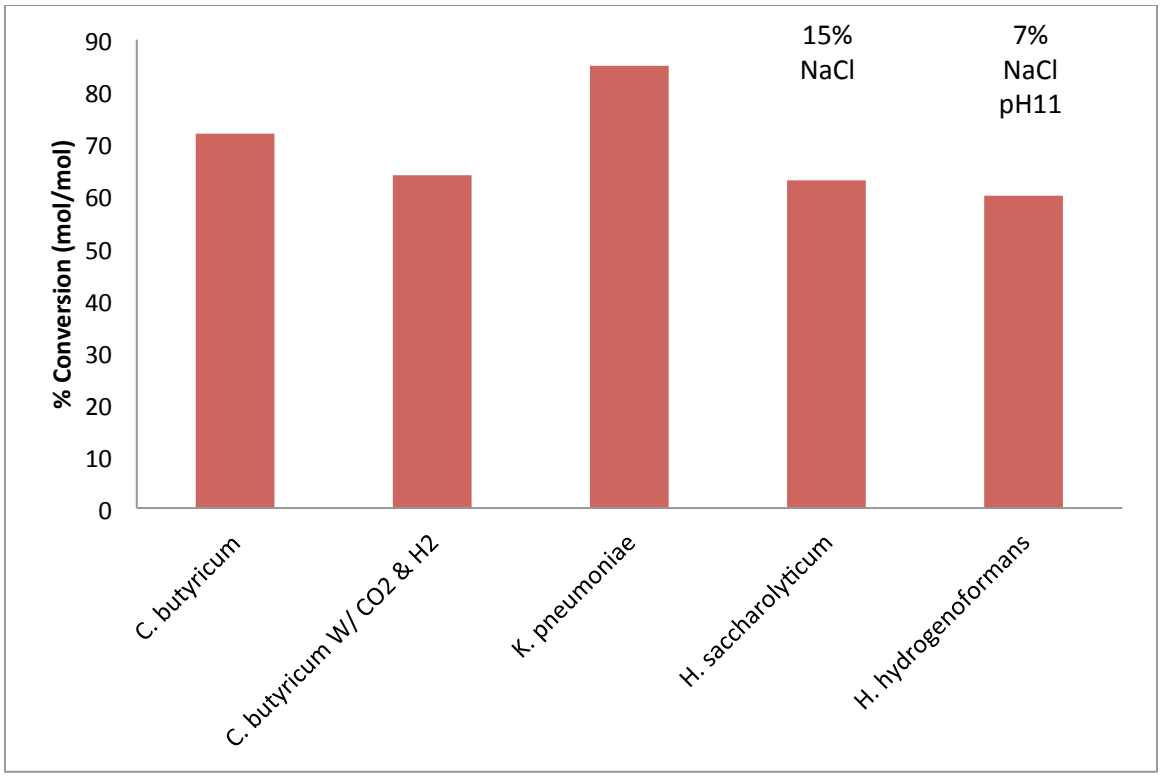

Figure 2.3. 1,3-propanediol yields from glycerol by four different organisms. *H. saccharolyticum* and *H. hydrogeniformans* were grown at 15% and 7% NaCl, respectively, and *H. hydrogeniformans* was also grown at a pH of 11.

2.2. GENOMICS OF HALANAEROBIUM HYDROGENIFORMANS

2.2.1. Genome Sequence. The genome of *H. hydrogeniformans* was completed in 2011 by using a combination of Illumina and 454 sequencing methods [4]. The genome consists of 2.6 mega bases, with four different rRNA operons. These 16s rRNA sequences were within 96% to 97% of other known *Halanaerobium* species, however, all of the currently identified species are neutrophilic, as compared to the alkaliphilic properties of *H. hydrogeniformans*. The genome contains 2491 theoretical proteins along with 98 transposase genes which consist of 4% of the total genome. This large transposon count could lead to potential problems in the future with regards to genetic manipulation.

2.2.2. Hydrogen Production. In anaerobic fermenters, hydrogen production is relegated to a ferredoxin exchange pathway associated with pyruvate metabolism [16]. When examining the genome of *H. hydrogeniformans*, an essential piece of this pathway appears to be missing. Ferredoxin hydrogenase, the final step in hydrogen production for anaerobic fermenters, has not been found within the genome. Using homolog sequence alignments, one NADP dependent dehydrogenase was identified, Halsa_1768, that had homology to other *Halanaerobium* ferredoxin hydrogenases. Further research will need to be conducted to identify the genes and regulatory mechanisms associated with the increased hydrogen production from *H. hydrogeniformans*.

2.2.3. 1,3-Propanediol Production and Vitamin B_{12}. The initial work with the 1,3-propanediol production pathway began with the genomic data. Through examination of the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways, it was identified that the organism had the potential to synthesize 1,3-propanediol from glycerol. The genome indicated that *H. hydrogeniformans* contained the required enzymes to carry out this conversion. *H. hydrogeniformans* possesses a glycerol dehydratase, Halsa_0984, and two 1,3-propanediol dehydrogenases, Halsa_0672 and Halsa_2285. Curiously, Halsa_2285 is 40 amino acids longer than Halsa_0672. However sequence alignment analysis has proven unfruitful in identifying the source of the extra 40 amino acids.

Figure 2.4 shows a KEGG pathway of the glycerolipid metabolism of *Halanaerobium hydrogeniformans* with the present genes highlighted in green.

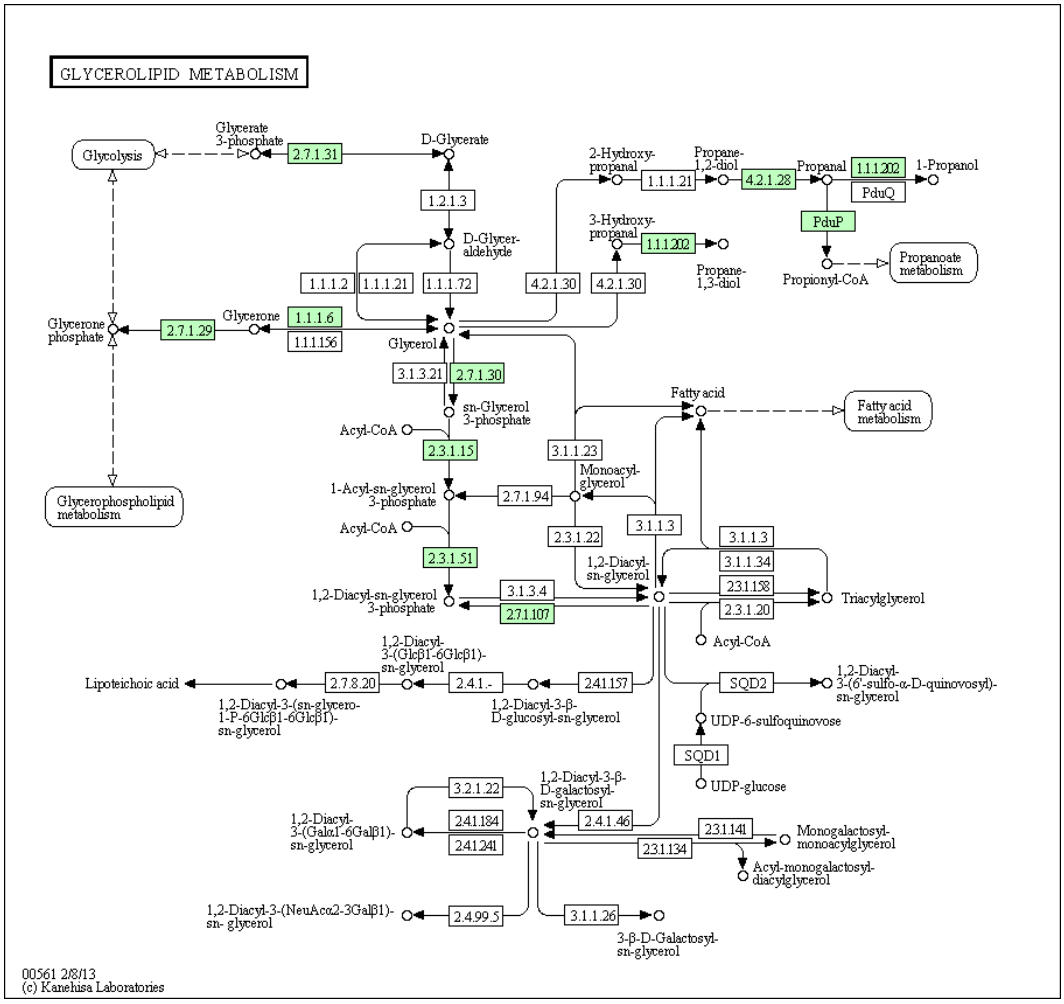

Figure 2.4. Glycerolipid Metabolism of *Halanaerobium hydrogeniformans*.

Another point of examination of the genome of *H. hydrogeniformans* is the synthesis of vitamin B_{12}. One of the biggest drawbacks to using anaerobic fermenters to

synthesize 1,3-propanediol is the need to supplement the media with vitamin B_{12}. Vitamin B_{12} is an essential cofactor for the first enzyme in the pathway, glycerol dehydratase. Many *Clostridia* species require the supplementation of vitamin B_{12} to obtain appreciable amounts of 1,3-propanediol production [5]. Most anaerobic fermenters cannot also produce their own vitamin B_{12}, or at least in amounts required for industrially significant 1,3-propanediol production. To test this, we examined the 1,3-propanediol production of *H. hydrogeniformans* in medium devoid of vitamin B_{12} supplementation. Surprisingly, we were able to obtain a conversion of approximately 30%, half of what was obtained in cultures supplemented with vitamin B_{12}. This lead to an examination of the vitamin B_{12} synthesis pathway within *H. hydrogeniformans* to determine if the organism was capable of producing Vitamin B_{12}. Figure 2.5 is a KEGG pathway of vitamin B_{12} synthesis and the enzymes present in *H. hydrogeniformans* are indicated by green boxes.

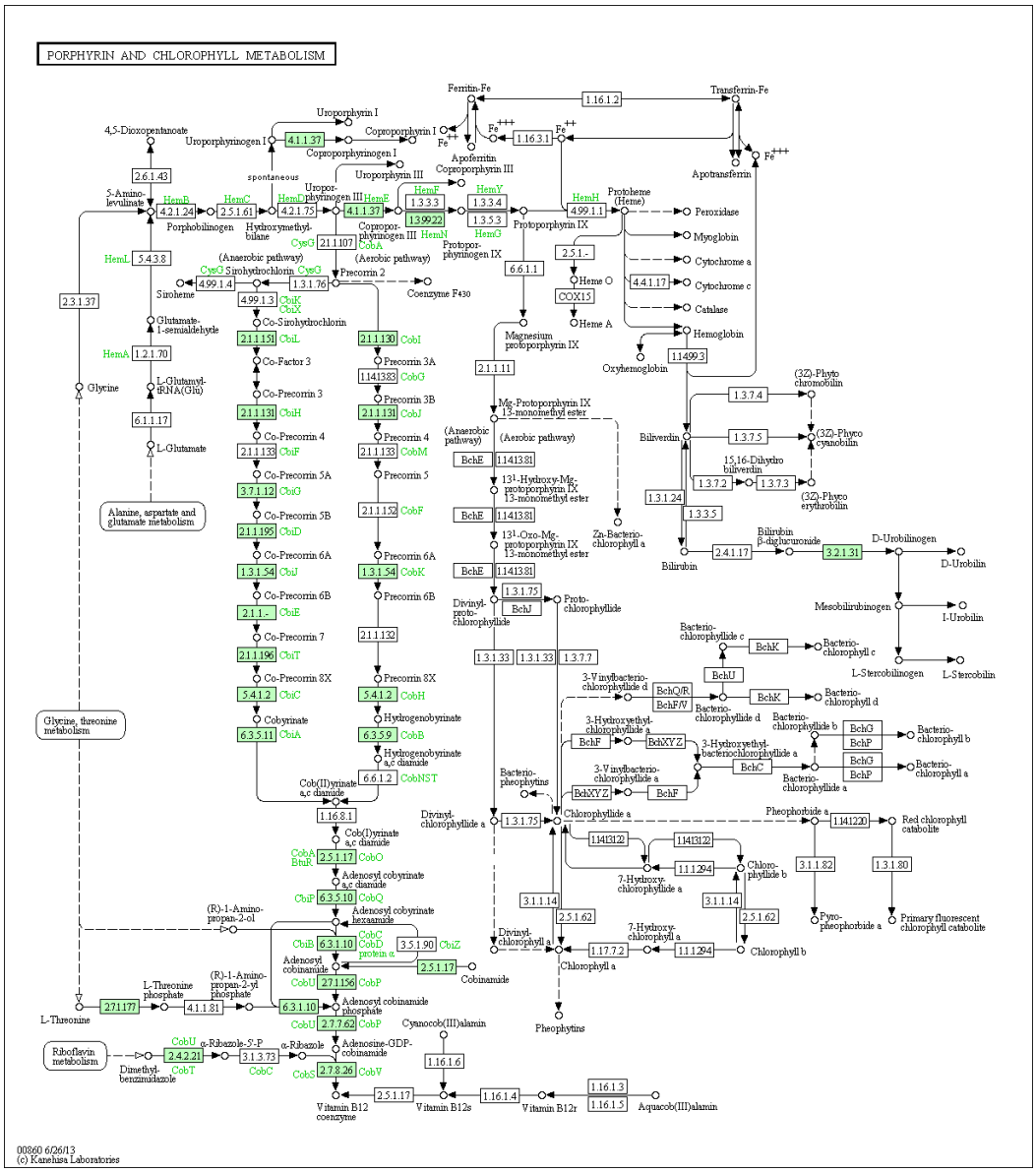

Figure 2.5. Vitamin B_{12} KEGG Pathway. The enzymes present in *H. hydrogeniformans* genome are indicated by green boxes. The pathway indicates a possible complete vitamin B_{12} synthesis pathway, possible providing another route for increased 1,3-propanediol production.

Interestingly, *Halanaerobium hydrogeniformans* contains an almost complete Vitamin B_{12} biosynthesis pathway. Further examination of the metabolic properties of *H. hydrogeniformans* will be required to better understand this synthesis pathway, production capabilities, and the effects of reduced vitamin B_{12} supplementation on 1,3-propanediol production. This line of research is essential in helping reduce the cost of microbial crude glycerol conversion, and bring this process into greater profitability.

PAPER

2. PRODUCTION OF 1,3-PROPANEDIOL FROM GLYCEROL UNDER HALOALKALINE CONDITIONS BY HALANAEROBIUM HYDROGENIFORMANS

To be submitted to the journal *Extremophiles*

2.1. ABSTRACT

With increased demands around the world to make modern lifestyles more environmentally friendly, the chemical commodity market has rapidly shifted. Through new technologies in chemical production, certain high value products have oversaturated the market and have become high-volume, low value waste products. The expansion of biodiesel production offers an excellent example; high volumes of glycerol byproduct from this process have shifted glycerol from a high priced commodity to a common waste product. Finding innovative biological processes to utilize these new waste products will help lead the way to new profitability and hydrocarbon independence. Some microorganisms are known to synthesize the polymeric precursor 1,3-propanediol from glycerol; however, crude glycerol from biodiesel production creates a harsh environment for most microbes, and must go through expensive pre-treatment steps to adjust pH and salt concentrations before it can be considered a suitable feedstock. *Halanaerobium hydrogeniformans* has been identified to convert glycerol into 1,3-propanediol under haloalkaline conditions. Samples were grown over five days at pH 11 and at 7% (w/v) sodium chloride. Vitamin B12 was used to stimulate 1,3-propanediol production. HPLC analysis indicated statistically significant production of 1,3-propanediol, with the vitamin B_{12} enhanced bottles having a significant increase in 1,3-propanediol production compared to glycerol-only samples. Data indicated a 0.6mol/mol conversion for vitamin B_{12} amended samples, while glycerol-only samples had a conversion rate of 0.32mol/mol. *H. hydrogeniformans*, and potentially other haloalkaliphilic bacteria, provides a unique opportunity to develop new chemical processes that can overcome traditional problems

and increase profitability by reducing the need for pH neutralization and dilution of residual salts in wastes such as crude glycerol.

2.2. INTRODUCTION

With the ever-expanding focus on utilizing waste products for chemical production, crude glycerol has become a target of recent research. Crude glycerol is generated from the production of biodiesel which is produced by utilizing animal or vegetable oils that are base-esterified, catalyzed by the addition of a strong base (sodium methoxide) and the addition of an alcohol (methanol) (Leung et al. 2010). The resulting products are biodiesel compounds and crude glycerol. The crude glycerol that is formed contains large amounts of methanol, sodium, and other contaminants (Yang et al. 2012). Due to the impurity of the glycerol, it is considered a waste product, and any technology that can utilize this waste at a minimum cost is a boon to companies big and small.

Potential applications of utilizing crude glycerol as a feedstock include the conversion of this waste product into value added chemicals and other fermentation products; one of the most profitable conversion products being the three carbon polyol, 1,3-propanediol. 1,3-propanediol is a polymer precursor predominantly used in paints, adhesives, and fragrances and is currently produced either through synthesis from propylene or ethylene oxide, or the biological conversion of glucose derived from corn (Yang et al. 2012). However, many anaerobic fermenters when metabolizing glycerol, produce 1,3-propanediol as a side product. 1,3-propanediol production is necessary as an NADH sink, and the organisms that conduct this process have to maintain a careful balance between growth and 1,3-propanediol production.

A unique extremophilic anaerobic fermenter that can conduct this process is *Halanaerobium hydrogeniformans*. This organism was isolated from Soap Lake, WA, and has been previously characterized to have optimal growth at pH 11 and 7% NaCl (w/v) (Begemann et al. 2012). Through genome identification, it was discovered that *H. hydrogeniformans* has the genetically capability to metabolize glycerol and produce 1,3-propanediol. This project's goal was to confirm this metabolic process and also examine

the role of vitamin B_{12} would have on the production capabilities of the bacterium, and finally explore tolerances and growth characteristics important for industrial application.

2.3. METHODS

2.3.1. Culture Conditions and Strain

The culture medium consisted of (per liter): 70g NaCl, 40g Na_2CO_3, 6.3g K_2HPO_4, 1g yeast extract, along with 10 ml of basal medium stock solution and 10 ml of trace mineral solution. The basal medium stock solution included (per liter): 50mg NH_4NO_3, 8.5mg $MgCl_2•6H_2O$, 7.5mg SiO_2, 4.5mg $MnSO_4•H_2O$, 4.2mg $CaCl_2•2H_2O$, 4mg methylene blue (as an oxygen indicator), and 1.8mg $FeSO_4•7H_2O$. The trace mineral solution included (per liter): 3g $MgSO_4•7H_20$, 1.63g Na_3-NTA, 1g NaCl, 0.64g $MnCl_2•4H_2O$, 0.13g $ZnCl_2$, 0.1g $FeSO_4•7H_2O$, 0.1g $CaCl_2•2H_2O$, $CoCl_2•6H_2O$, 0.03g $NiSO_4•6H_2O$, 0.025g $Na_2MoO_4•2H_2O$, 0.025g $Na_2WO_4•2H_2O$, 0.01g $AlK(SO_4)_2•12H_2O$, 0.01g H_3BO_3, and 7mg $CuCl_2•2H_2O$.

Anaerobic cultures were prepared in 160mL serum bottles. The medium was prepared by boiling to degas under a N_2 blanket. As the medium cooled, reductant stock mix was added to the media that contained 0.75g Na_2S and 0.6g cysteine per liter. Once the media was cooled, the flasks were transferred into a Coy anaerobic glove bag where the 50mL of media was dispensed into 160mL serum bottles filled and autoclaved (121°C, 20min). After autoclaving, the headspace gas was exchanged for 80%N_2/20% CO_2 mixture. The bottles then were inoculated with a 10% inoculum from previous stock cultures.

30 mM glycerol or crude glycerol were added to the medium unless otherwise stated, along with 1,3-propanediol for the tolerance experiments. Vitamin B_{12} supplementation from anaerobic, filter-sterilized stocks were added right before inoculation.

2.3.2. Sampling and Protein Analysis

Samples were taken every 24 hours, unless specified differently. 5mL syringes were degassed with N2/CO2 mix, and 1 mL of culture sample was removed for each of the sample periods. The sample was placed in a 1.5mL Eppendorf tube, and centrifuged for 5 min at 13000 x g. The supernatant was decanted into another 1.5mL Eppendorf tube, and frozen for HPLC analysis. The remaining protein sample had 1mL of filter-sterilized water added to them to bring them to the original sample volume.

For protein analysis, protein samples were amended with 0.3 mL of a 5N NaOH solution, and held at 90°C for 30 min. The samples were then prepared following the guidelines of the BIORAD BCA assay kit.

2.3.3. HPLC Analysis

Filter sterilized samples (.45 μM PTFE filters) were injected onto a 300 x 7.8 mm Aminex HPX-87H column (BioRad, Hercules, CA) maintained at 50^{o}C. The mobile phase was 2.5 mM H_2SO_4 maintained at a constant flow rate of 0.6 ml/min and at approximately 2.2 MPa. Detection was done with both a UV 231 (at 210 nm) and refractive index monitor.

2.4. RESULTS

2.4.1. Glycerol and 1,3-Propanediol Tolerances

An experiment was conducted to examine the tolerance of *H. hydrogeniformans* to concentrations of glycerol. A gradient was prepared ranging from 0 mM glycerol up to 1920 mM glycerol. Growth was examined through turbidity readings and protein analysis. *H. hydrogeniformans* was capable of growth at 7.5, 15, 30, 60, 120, 240, 480, 960, and 1920 mM glycerol. It did not exhibit any growth without glycerol in the medium. Next, 1,3-propanediol tolerance of *H. hydrogeniformans* was determined. A gradient was prepared very similar to the glycerol tolerance, however the maximum

conversion of glycerol to 1,3-propanediol under ideal conditions is 75%. Thus, it was expected that the tolerances would correspond to a 1,3-propanediol concentration for a 75% conversion. The bottles were amended with 30mM glycerol as a carbon source. H. hydrogeniformans was capable of growth with 1,3-propanediol amendments of 0, 10, 30, 60, 120, and 380 mM. There was no growth evident at an 1,3-propanediol amendment of 750 mM.

2.4.2. 1,3-Propanediol Production

The production capabilities of *H. hydrogeniformans* and the influence of vitamin B_{12} supplementation were studied. A gradient was prepared to examine the maximum production of 1,3-propanediol from media containing 30mM glycerol. Approximately 16.5 mM 1,3-propanediol was produced when the culture was amended with 25, 50, 75, or 100 μg/L vitamin B12 and approximately 8.5 mM 1,3-propanediol when no vitamin B12 was provided (Figure 2.1). Table 2.1 indicates the percent mole/mole conversion of glycerol to 1,3-propanediol in *H. hydrogeniformans* cultures when supplemented with vitamin B_{12}.

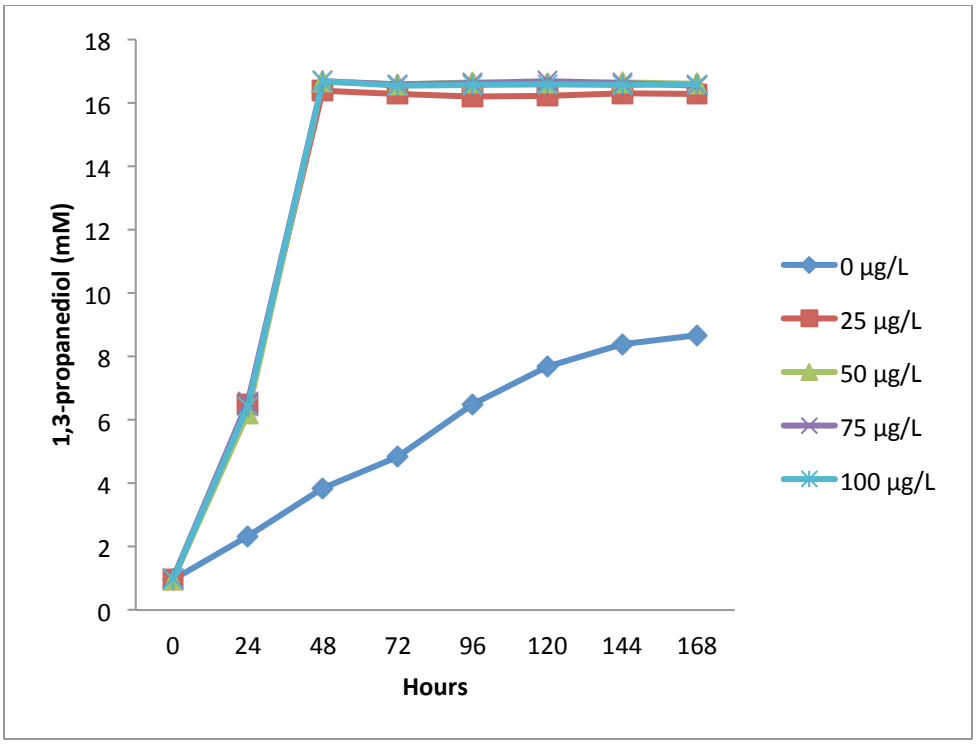

Figure 2.1. 1,3-propanediol production by *H. hydrogeniformans* and Vitamin B_{12} role.

Table 2.1. Percent mole/mole conversion of glycerol to 1,3-propanediol in H. hydrogeniformans cultures supplemented with vitamin B12.

B_{12}	**% (mol/mol)**
0 µg/L	31.5
25 µg/L	59.1
50 µg/L	60.3
75 µg/L	60.1
100 µg/L	60.2

2.4.3. Crude Glycerol Tolerance

The growth of *Halanaerobium hydrogeniformans* was examined under both 0.1% and 0.5% crude glycerol. In both instances, growth was indicated.

2.5. DISCUSSION

Examining glycerol tolerance is a first step in developing a potential industrial process with *H. hydrogeniformans*. Our growth experiments show that *H. hydrogeniformans* can tolerate at least 2M glycerol concentrations in addition to the already extreme conditions of the growth medium. First, the salt uptake mechanism many *Halanaerobium* species utilize for halotolerance may be stabilizing the osmotic pressure (Oren 2009). Previous studies have also shown that *H. hydrogeniformans* can tolerate growth conditions in at least 15% NaCl (Begemann et al. 2012), the growth conditions in this experiment contained 18% glycerol and 7% NaCl, which may indicate other influences in the tolerance. Glycerol could be inhibiting growth, however the organism may be able to deplete the high concentrations of glycerol in the media through other metabolic pathways, allowing for eventual growth tolerance..

Tolerance to the fermentation product is also of great concern when developing a process for industrial applications. Our current *H. hydrogeniformans* strain can tolerate at least 0.38M 1,3-propanediol, which is 75% of the max theoretical yield when no gas is produced during fermentation, and 60% of the maximum yield when hydrogen and carbon dioxide are produced (Zeng 1996). These tolerances are expected to increase through subsequent culturing and acclimation of the culture to high concentrations of 1,3-propanediol. Subsequently, examination of tolerance in a continuous feed bioreactor will give a better representation of the real world values in an industrial process.

When examining the production capability of *H. hydrogeniformans* unexpected results occurred. The first identification of this process was in June of 2012. At that time, we were able to obtain a conversion percent of approximately 5% without B_{12} and 47% with B_{12} (Roush et al. 2012). A year later, in May of 2013, we can now show that the organism is able to do this conversion without B_{12} at 33%, and with the

supplementation of B_{12} at 60%. These increases over a year can likely be attributed to selection pressures within our growth media

In summary, we have identified *Halanaerobium hydrogeniformans* as a capable glycerol fermenter under haloalkaliphilic conditions. *H. hydrogeniformans* can tolerate concentrations of glycerol of 2M, and 1,3-propanediol concentrations of .38M. When converting glycerol to 1,3-propanediol, *H. hydrogeniformans* has a maximum conversion of 60% with Vitamin B_{12} supplementation, and 31.5% without B_{12}. *H. hydrogeniformans* can also grow and thrive in samples of crude glycerol. Our work has shown that *Halanaerobium hydrogeniformans* is a capable glycerol fermenter with potential viability for new biotechnological processes.

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SECTION

3. CONCLUSIONS

Exploring extremophiles for biotechnology provides an exciting and underdeveloped avenue for biotechnology and industrial microbiology. Our work with *Halanaerobium hydrogeniformans* in haloalkaliphilic conditions has given light potential of extremophiles in industrial applications, along with providing insight into unique metabolic characteristics of newly discovered organisms. As extremophilic biotechnology begins to expand in the coming years, scientists will be able to better understand the true physiological limits of microorganisms.

In summary, we have identified *Halanaerobium hydrogeniformans* as a capable glycerol fermenter under haloalkaliphilic conditions. *H. hydrogeniformans* can tolerate concentrations of glycerol of 2M, and 1,3-propanediol concentrations of .38M. When converting glycerol to 1,3-propanediol, *H. hydrogeniformans* has a maximum conversion of 60% with Vitamin B_{12} supplementation, and 31.5% without B_{12}. *H. hydrogeniformans* can also grow and thrive in samples of crude glycerol. Our work has shown that *Halanaerobium hydrogeniformans* is a capable glycerol fermenter with potential viability for new biotechnological processes.

Future work with *Halanaerobium hydrogeniformans* will focus on developing the glycerol fermentation pathway for industrial implementation. Many variables need to be examined, like methanol tolerance, reaction rates, and antibiotic resistance. Many of the experiments conducted during this work focused on finding limits and growth conditions suitable for 1,3-propanediol conversion. The next steps involve scaling this process to bioreactor scale, and examining how *Halanaerobium hydrogeniformans* and 1,3-propanediol production occur under conditions most similar to industrial settings.

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VITA

Daniel Roush was born in Jefferson City, Missouri. He has earned a Bachelor of Science degree at Missouri University of Science and Technology in 2011. During his time at Missouri S&T, Daniel worked with the International Genetically Engineering Machines team, focusing on bioenergy production and microbial fuel cells. He held many offices, including president and presided over the executive board that had iGEM officially recognized as an organization by the University. His undergraduate research was split between working with iGEM and also working under the guidance of Dr. Dave Westenberg. After completing his undergraduate degree, Daniel joined Dr. Melanie Mormile's lab in the fall of 2011 to begin work on his master's degree. Daniel earned his Master of Science degree in Applied and Environmental Biology from the Missouri University of Science and Technology in the fall of 2013. Daniel began his PhD work at Arizona State University in the fall of 2013, with a focus on extremophiles and microbial ecology.